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Review Article

Development of therapeutic genome engineering in laminin-α2-deficient congenital muscular dystrophy

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Muscular dystrophies are a heterogeneous group of genetic muscle diseases that are often characterized by pathological findings of muscle fiber degeneration and the replacement of muscle fibers with fibrotic/connective tissues. In spite of the genetic causes of many of these conditions having been identified, curative treatments are still lacking. Recently, genome engineering technologies, including targeted gene editing and gene regulation, have emerged as attractive therapeutic tools for a variety of muscular dystrophies. This review summarizes the genome engineering strategies that are currently under preclinical evaluation for the treatment of LAMA2-deficient congenital muscular dystrophy. In particular, we focus on the applications of CRISPR/Cas9 to correct a splice site mutation in *LAMA2* and to up-regulate a disease-modifying gene *LAMA1*. Finally, the challenges faced in the clinical translation of these strategies are discussed.

Introduction

Congenital muscular dystrophy (CMD) represents a diverse group of early-onset muscle disorders characterized by hypotonia or low muscle tone, which begins at infancy or very early childhood (typically before the age of 2). There are 11 subtypes of CMDs mapped to over 30 genetic loci (recently reviewed in [1]). Mutations in the *LAMA2* gene causes type 1A CMD, also known as merosin-deficient congenital muscular dystrophy type 1A (MDC1A; OMIM 607855). It affects ∼1–9 in 1 000 000 individuals worldwide and is inherited in an autosomal recessive manner.

The *LAMA2* gene encodes the extracellular protein laminin-α2, which polymerizes with laminin-β1 and -γ1 to form the laminin-211 heterotrimeric complex. Laminin-α2 has multiple binding partners such as integrin α7β1 and α-dystroglycan that are collectively required for adhesion, basement membrane assembly, and a multitude of signaling events that protect skeletal muscle from damage (Figure 1). Therefore, the lack of laminin-α2 in individuals with MDC1A manifests as progressive muscle weakness and degeneration, the accumulation of connective or fibrotic tissues, as well as peripheral neuropathy and white matter abnormalities. The clinical severity of the disease highly correlates with the degree of laminin-α2 deficiency. Individuals with mutations that result in the complete absence of the laminin-α2 chain never achieve independent ambulation, requiring enteral feeding and ventilator support, while partial deficiency results in a milder disease phenotype.

Similar genotype–phenotype correlations are also observed in the MDC1A mouse models. The dy^W/dy^W mice express negligible amounts of a truncated laminin-α2 chain and develop severe dystrophic phenotypes, which are even more exaggerated in the *Lama2* null dy^{3K}/dy^{3K} mice. In contrast, the dy^{2j}/dy^{2j} mice, that express only slightly reduced levels of truncated laminin-α2 chain, exhibit milder symptoms of muscular dystrophy. Therefore, the restoration of laminin-α2 expression holds tremendous potential to ameliorate muscular dystrophy in MDC1A. However, standard gene therapy

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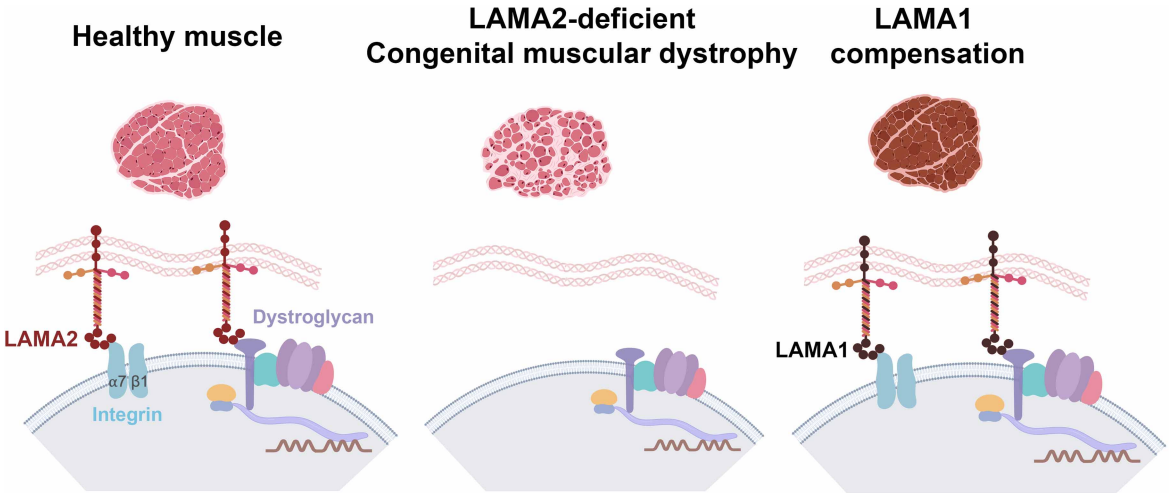

Figure 1. Schematic overview of skeletal muscle histology and proteins in healthy and dystrophic conditions.
LAMA2-deficient congenital muscular dystrophy type 1A (MDC1A) is caused by the lack of LAMA2 protein, a crucial component in the skeletal muscle membrane. Up-regulation of similarly structured LAMA1 protein compensates for the lack of LAMA2 and improves muscle pathophysiology in MDC1A mouse models.

approaches to reintroduce *LAMA2* cDNA into the genome are hampered by the large size of the laminin-α2 protein, which exceeds the packaging capacity of a clinically relevant delivery vehicle such as the adeno-associated virus (AAV). Antisense oligonucleotide-mediated exon skipping can restore expression of laminin-α2 and was found to increase survival when similarly targeted to remove an artificial neo-cassette in the *Lama2* exon 4 in the dy^{3K}/dy^{3K} mice [2,3]. Critically, these findings support the notion that the rescue of laminin-α2 can be beneficial in improving disease pathology, given a technology to do so.

An alternative and promising therapeutic strategy is to compensate for the loss of laminin-α2 with the structurally similar protein, laminin-α1, which can also form a heterotrimeric structure with β1 and γ1 chains, creating the laminin-111 complex (Figure 1). Laminin-α1 is encoded by the *LAMA1* gene, which shares ∼80% sequence similarity to *LAMA2*. However, the laminin-α1 expression is normally restricted to epithelial cells and is absent from postnatal skeletal muscle and nerves. Yet, transgenic overexpression of *Lama1* ameliorates muscle wasting and paralysis in the MDC1A mouse models [4–6]. In addition, recombinant laminin-111 protein supplementation enhances muscle regeneration and improves muscle function and survival rate in the dy^{W}/dy^{W} mice [7,8]. Together, these studies strongly support the compensatory potential of the gene. Still, the large size of *LAMA1* is a significant barrier not only to traditional gene therapy approach but also to producing sufficient quantities of recombinant protein (recently reviewed in [9]).

Collectively, the challenges undermining the therapeutic potential of correcting disease-causing mutations in *LAMA2* or up-regulating *LAMA1* provide an excellent opportunity to put technologies such as genome engineering to the test.

Utilization of CRISPR/Cas9 as a genome engineering technology

The discovery of genome engineering technologies and a variety of nucleases has enabled precise modification of the mammalian genome, including the correction of pathogenic mutations. Genome editing involves the introduction of an engineered nuclease, leading to the generation of a double-strand break (DSB) at a desired location in the genome, followed by an endogenous DNA repair process through either non-homologous end-joining (NHEJ) or homologous-directed recombination (HDR). NHEJ is the dominant DNA repair pathway that occurs in all phases of the cell cycle, whereas homologous recombination involves the copying of DNA from a homologous template, which only occurs in the late S- or G2 phase of the cell cycle and accordingly occurs with much less frequency. Nevertheless, HDR is considered to be error-free, as opposed to NHEJ that is

known to introduce the insertion or deletion (indel) of random nucleotides at the DSB site and, therefore, is generally considered to be error-prone. However, this view is often too simplistic, since error-free events occur at high frequency in mammalian cells, challenging the fallibility of NHEJ [10,11].

The most common engineered nucleases that rely on specific protein domains to target genomic DNA are zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) (previously reviewed in [12]). The ZFNs are fusion proteins comprising an array of site-specific DNA-binding domains attached to the endonuclease domain of the bacterial FokI restriction enzyme. Each domain recognizes a triplet (3 bp) DNA sequence, and tandem dimerized domains can potentially bind to an extended nucleotide sequence (9–18 bp) that is unique within the genome. In contrast with the triplet requirement of the zinc finger domain, each TALE domain recognizes a single nucleotide and can be flexibly extended to the desired length of the genomic target. Nevertheless, both technologies require re-engineering a new protein for each target, contributing to the challenge for genome alteration and/or clinical application.

The more recently discovered genome engineering tool, the CRISPR/Cas9, utilizes a DNA-targeting mechanism that is programmed through Watson–Crick RNA–DNA pairing rather than protein–DNA interaction. A single-guide RNA (sgRNA) is required to direct the Cas9 nuclease to the target DNA sequence, where a DSB can occur. The only design limitation in this system is the requirement for a protospacer adjacent motif (PAM) sequence recognized by Cas9. Depending on the species of origin and modification, several PAM sequences have been described, including NGG and NAG (*Streptococcus pyogenes*), NGA (*S. pyogenes* EQR variant), NGCG (*S. pyogenes* VRER variant), NNGRRT (*Staphylococcus aureus*), NNNRRT (*S. aureus* KKH variant), NNAGAA (*Streptococcus thermophilus*), NNNNRYAC (*Campylobacter jejuni*), and YG (*Francisella novicida*) [13–19]. The different PAM recognition sequences further broaden the targeting flexibility of Cas9.

An alternative use of the CRISPR/Cas9 technology with potentially broader applications is not to cut DNA, but rather to change the level or regulation of gene targets to achieve therapeutic outcomes. CRISPR/Cas9 has been repurposed as a genome-docking platform through alteration of its catalytic residues to create a nuclease-deactivated Cas9 (dCas9), which is unable to cleave DNA but still retains its ability to bind to specific genomic sequences. The dCas9 can be further coupled with transcriptional repressors (e.g. KRAB domain) or activators (e.g. VP64) and engineered into a programmable artificial transcription factor in eukaryotic cells.

In this review, we will outline our efforts to exploit CRISPR/Cas9 and CRISPR/dCas9 technologies as targeted nucleases and transcriptional activators for the potential treatment of MDC1A (Figure 2).

CRISPR/Cas9-mediated mutation correction in MDC1A

The dy^{2j}/dy^{2j} mice express a residual level of a truncated form of laminin-α2. Early biochemical characterization and DNA sequencing of this mouse model revealed a g→a point mutation in the first nucleotide in intron 2 (*Lama2* c.417 + 1g→a), which is a donor splice site of the second exon in *Lama2* [20]. This single base mutation results in spontaneous exon 2 skipping, leading to the disruption of N-terminal domain of the protein. Of note, the reading frame of *Lama2* transcript in the dy^{2j}/dy^{2j} mouse model is preserved, supporting the notion that exon skipping may not be an optimal treatment strategy for MDC1A.

Precise gene modification relying on the HDR pathway can theoretically correct the majority of pathogenic point mutations, including the splice site mutation found in the dy^{2j}/dy^{2j} mice. However, since HDR is extremely inefficient in post-mitotic tissues including skeletal muscle, the development of alternative strategies to correct mutations causing muscular dystrophy is necessary.

We recently described the utilization of the NHEJ pathway to correct the splicing defect in the dy^{2j}/dy^{2j}*i*-mouse model (Figure 2a) [21]. We analyzed the region surrounding the disease-causing splice site (+1 position) mutation for Cas9 cleavage sites at the exon–intron junction. Afterwards, the downstream intronic region was evaluated for the concurrent cleavage prior to 'gt' sequences (splice site-generating cut), which is part of a nine-nucleotide consensus sequence found in mammalian cells that is crucial for normal splicing (A/C-A-G-g-t-a/g-a-g-t) [22].

In a proof of principle study, we showed that the combination of Cas9 and sgRNAs targeting these intronic sections of the *Lama2* gene created simultaneous DSBs in the genomic region flanking the two targets. NHEJ-mediated repair of the breaks successfully reconstituted a functional splice donor site in *Lama2*, leading to correction of the splicing defect and restoration of Lama2 protein expression.

When introduced into neonatal dy^{2j}/dy^{2j} mice using AAV9, restoration of laminin-α2 protein in its full-length version markedly improved muscle functions and histopathology. In addition, the utilization of AAV9 targets skeletal muscle and peripheral nerves well, resulting in near complete amelioration of phenotypes in

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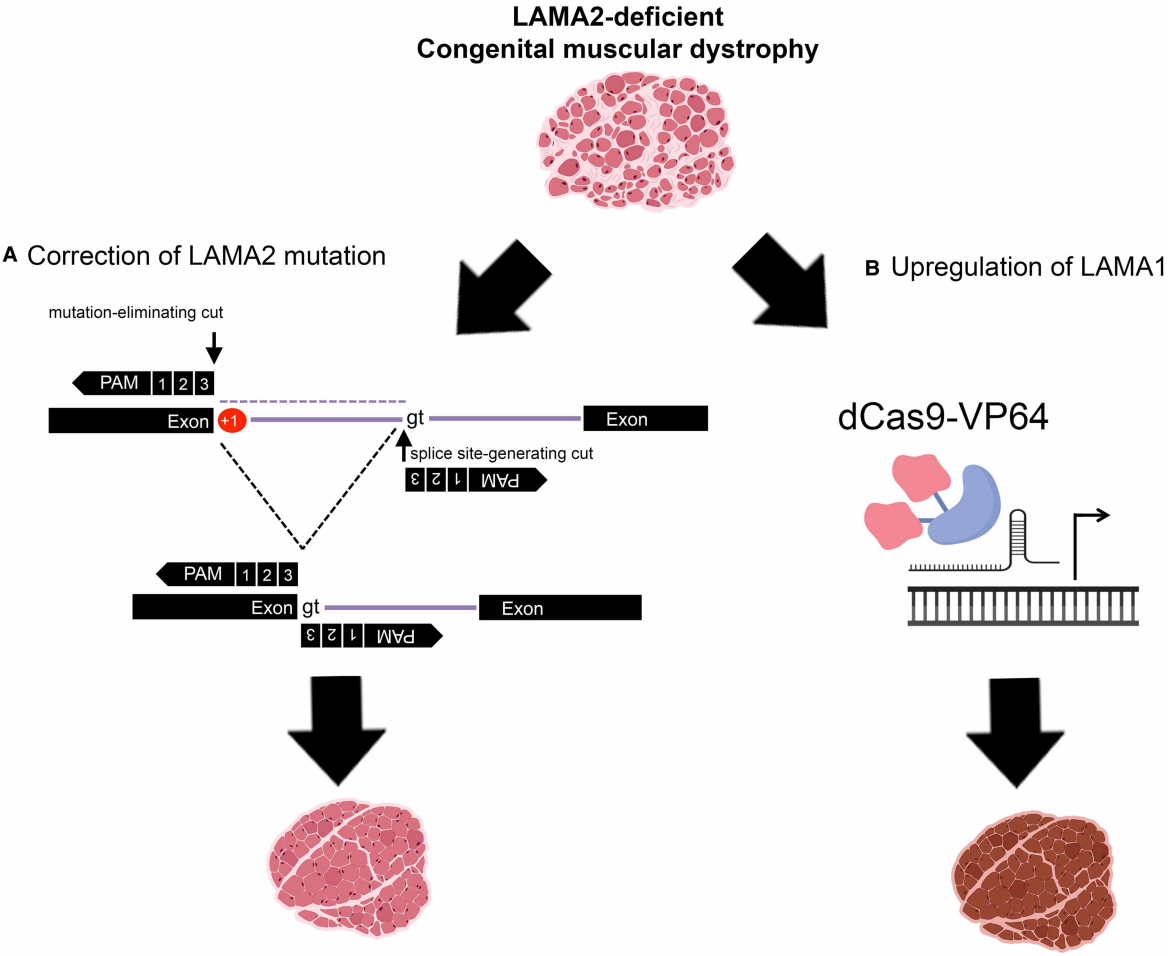

Figure 2. Genome engineering-based therapeutic strategies for MDC1A.

The potential therapeutic application of CRISPR/Cas technology involves correction of *LAMA2* splice site mutation (**a**) and up-regulation of compensatory gene *LAMA1* (**b**). The dy^{2J}/dy^{2J} mouse model harbors a disease-causing splice site (+1 position) mutation, resulting in non-functional donor splice site. To correct this mutation, the region surrounding the mutation was analyzed for Cas9 cleavage sites at the exon–intron junction. Afterwards, the downstream intronic region was evaluated for the concurrent cleavage prior to 'gt' sequences (splice site-generating cut). Combination of Cas9 and sgRNAs created simultaneous DSBs in the flanking region. Following NHEJ-mediated repair, a functional splice donor site is reconstituted, leading to correction of the splicing defect and improvement in muscle histopathology. In (**b**), sgRNAs were designed to target the proximal promoter region of *Lama1*, immediately upstream of the transcription start site (TSS). The endonuclease catalytic residues of Cas9 were mutagenized to create deactivated Cas9 (purple, dCas9) and then fused to transcriptional activators VP64 (pink). Combination of dCas9, VP64, and sgRNas targeting *Lama1* increased transcript expression and protein, leading to improvement in dystrophic phenotypes.

both tissues. While it is encouraging, it remains to be seen whether an improvement to a similar degree may be achieved when the treatment is given at early- and/or post-symptomatic ages.

There are more than 300 disease-associated mutations identified in the *LAMA2* gene, which include a significant number of variants affecting canonical donor splice sites [23,24]. Correction of splicing defects may be extended to many of these mutations, given that sgRNA pairs were designed for each variant. Furthermore, with future discoveries and directed engineering of additional Cas9 species with a variety of PAM sequences, the approach of correcting noncoding mutations by modifying an intronic region to create a functional splice donor site may be applicable to various inherited diseases.

CRISPR/dCas9-mediated modulation of a compensatory gene in MDC1A

Given the heterogeneity of MDC1A-causing genomic alterations, CRISPR/Cas9-mediated gene-editing strategies would require the design and thorough analysis of multiple sgRNAs specific for each individual mutation. The correction approach described in the previous section only applies to patients with mutations affecting splice donor site. Moreover, safety concerns regarding CRISPR/Cas9's potentially mutagenic nature and the presence of off-target effects after gene editing remain, which together may prove to be challenging from a safety and regulatory point-of-view.

An alternative approach that enables the treatment of more patients, irrespective of their mutation types, is specific up-regulation of compensatory proteins. The aforementioned laminin-α1, encoded by the *LAMA1* gene, represents the most promising compensatory mechanism for the lack of *LAMA2*. Despite significant therapeutic benefits in mice, it is important to realize that transgenic approaches are not clinically feasible to humans because it would entail modifying the genome from the embryonic stage. Therefore, we opted to up-regulate *Lama1* postnatally using dCas9, coupled with 2XVP64 transcriptional activator domains. This construct was guided to the *Lama1* proximal promoter region by a combination of three sgRNAs. We showed that systemic administration of AAV9-carrying the CRISPR/dCas9 components led to robust up-regulation of laminin-α1 in the dy^{2j}/dy^{2j} mice, resulting in the amelioration of dystrophic features in skeletal muscle and improvement in mobility and muscle functions of the mice [25].

Furthermore, by altering the sgRNAs to target the human rather than the mouse *LAMA1* promoter, we were able to demonstrate up-regulation of *LAMA1* in MDC1A patient-derived fibroblasts (Kember et al., manuscript in preparation). Collectively, the application of CRISPR/dCas9 transcriptional up-regulation of a disease modifier gene such as *LAMA1* has the potential to be applied to all MDC1A patients, irrespective of their mutations. Additionally, utilization of nuclease-deactivated Cas9 that is unable to cleave DNA may bypass the potential risks about the unintended off-target genomic alteration.

Concluding remarks and future perspectives

Genome engineering tools have been widely adopted for therapeutic approaches, including by the neuromuscular disease field. Nevertheless, many challenges still remain before fully realizing the potential of genome engineering for gene therapy.

With respect to the genome-editing approach to correct splice site mutations, the first challenge involves the heterogeneity of the pathogenic variants found in patients. We computationally predicted that the exon inclusion strategy might be applicable to more than 47% of disease-causing splice site mutations beyond MDC1A [21]. Nevertheless, a customized CRISPR/Cas9 strategy tailored to each mutation would ultimately need to be designed and tested. Trials involving rare disease patients are limited by low numbers of affected individuals, inadequate follow-up, and lack of randomization or binding, therefore, will require a paradigm shift from a regulatory point-of-view.

Another important aspect to consider is the safety pertaining to potential off-target effects. Successful application in diverse organisms demonstrates that the technologies are effective with limited negative side effects *in vivo*. However, the translation of these tools to clinical settings requires analysis at a greater resolution for off-target effects than has previously been attempted. In this regard, rapid advances are being made for increasing the specificity of genome-editing tools, including the use of truncated guide RNA molecules [26,27], new variants or orthologues of Cas9 nucleases with higher fidelity [16,28,29], as well as the development of unbiased approaches for detecting off-target effects with a much greater level of sensitivity compared with previous methods [30–32]. However, the detection limits of these analyses are still restrained by the accuracy of current DNA sequencing technologies, and it will be a critical area for improvement moving forward. In addition, it remains uncertain whether all off-target effects can be accounted for in a therapeutic setting that targets one site within billions of DNA base pairs, involves modification of millions of cells and is custom prepared for each patient. It may also be important to account for genetic variation between individuals, as well as the impact of common variants in the population. Scott and Zhang analyzed [33] data sets from Exome Aggregation Consortium (ExAC) and 1000 Genomes to determine how human genetic variation affects target choice for Cas endonucleases in the context of therapeutic genome editing. They found that in large populations of individuals, most candidate off-target sites will be rare, underscoring the need for prescreening of patients through whole-genome sequencing to ensure safety. In the future, this information may be integrated

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with empirical methods for guide RNA selection into a framework for designing CRISPR-based therapeutics that maximizes efficacy and safety across patient populations. Therefore, it may become routine to check the genome-wide activity of CRISPR/Cas9-mediated editing in the context of an individual's specific genome.

Alternative technologies that do not rely on the creation of DSBs such as the CRISPR activation approach largely bypass the concerns about mutation heterogeneity and off-target cleavage. In the context of MDC1A, in which the types of mutations are heterogeneous, the CRISPR/dCas9-mediated activation of a compensatory gene circumvents the need to design mutation-specific genome-editing approaches. Moreover, many rare diseases correlate with too little functional gene-product that alters protein activity or haploinsufficiency, in which CRISPR activation may serve as a potential treatment modality [34,35]. Similarly, epigenome editing that specifically changes gene regulation through modifications to chromatin structure is also creating new ways to manipulate the genome for therapeutic purposes [36].

As Cas9 originates from prokaryotic cells, its expression may cause cytotoxicity and trigger immune responses in mammalians. Although expression of CRISPR/Cas9 components for as long as 30 weeks did not cause obvious tissue damage in our recent study, we did not evaluate the mice for immune responses [21]. Cellular and humoral immune responses in mice induced by AAV-delivered Cas9 following intramuscular administration have been observed [37]. Furthermore, the presence of pre-existing adaptive immune responses to Cas9, both of *S. aureus* and *S. pyogenes* origin, were detected in human sera [38–40], which may not only prevent the *in vivo* expression CRISPR components but also to induce tissue or organ damage due to a cytotoxic T-cell response. Although more studies are needed, cautions should be exercised and more work needs to be done before *in vivo* CRISPR genome editing can be used in humans.

Another important aspect that must be addressed involves the delivery to tissues of interest. The AAV vectors are highly promising therapeutic gene delivery vehicles that also offer the potential to facilitate and enhance many clinical applications of genome engineering, yet numerous hurdles must still be overcome. Concerns over the long-term safety of delivering genome-modifying cargo to cells and host immune responses to the AAV vectors and the cargo must also be addressed. In the case of MDC1A, amelioration of disease phenotypes *in vivo* highly depends on efficient targeting in both the skeletal muscles and peripheral nerves [21,25]. Improvements of the biodistribution, tissue penetration and, in particular, editing efficiency are required in order to fully exploit the potential of therapeutic AAV delivery. Furthermore, due to the size constraints associated with AAV vectors, it also remains unknown whether dual-particle delivery or the use of an AAV-split-Cas9, which may be necessary for certain applications, can support sufficient levels of modification to yield a therapeutic effect [37]. As an alternative, nonviral approaches to introduce CRISPR/Cas9 that are currently being studied show the potential to address many of the aforementioned limitations with viral vectors (recently reviewed in [41]).

Finally, as with any other therapeutic modality, time of intervention is a critical part to determine the feasibility of translation to humans. Often, preclinical studies test a therapeutic approach administered very early in the disease progression or prior to the onset of symptoms [21]. Most patients with neuromuscular disorders are diagnosed after the onset of symptoms, and these are going to constitute the most common patient population. Yet, these are the patients who are likely to receive a relatively modest benefit instead of the more dramatic rescue seen at earlier ages. This benefit could still be significant and meaningful as it may have the potential to improve the overall quality of life. It also presents a rationale for the implementation of better diagnostic procedures such as newborn screening to identify patients before the onset of symptoms, when a treatment will be maximally effective.

In summary, there is still a long way to go in order to fully exploit the potential of genome engineering in translational medicine. While preclinical studies must address many issues outlined here, there is no reason to believe that any such challenges cannot be surmounted. Advances in genomic technology will pave the way for safe and effective CRISPR/Cas9-based therapies.

Summary

- The versatility of CRISPR/Cas9 technology allows the correction of causative mutations in human diseases. We utilized the technology to simultaneously remove a point mutation and create a functional donor splice site in *Lama2* gene, thus demonstrating its therapeutic benefit in the MDC1A mouse model. Correction of splicing defects in this way may be extended to various inherited diseases.

- The application of CRISPR/dCas9 transcriptional up-regulation of a disease modifier gene such as *LAMA1* has the potential to be applied to all MDC1A patients, irrespective of their mutations.
- Fundamental studies such as the nature and biology of basement membranes, combined with the advancement gene therapy and CRISPR/Cas9, contribute to a considerable progress in the development of treatments for MDC1A.
- Similar to many other therapeutic modalities, the safety aspects concerning off-target effects, cytotoxicity and immune responses, as well as finding optimum window of intervention and delivery methods require further studies. Overall, it should not minimize the enthusiasm to move CRISPR/Cas9-based therapies closer towards clinical application.

Abbreviations

AAV, adeno-associated virus; CMD, congenital muscular dystrophy; DSB, double-strand break; ExAC, Exome Aggregation Consortium; HDR, homologous-directed recombination; MDC1A, merosin-deficient congenital muscular dystrophy type 1A; NHEJ, non-homologous end-joining; PAM, protospacer adjacent motif; sgRNA, single-guide RNA; TALENs, transcription activator-like effector nucleases; TSS, transcription start site; ZFNs, zinc finger nucleases.

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Competing Interests

The authors have filed patent applications related to application of genome editing for rare diseases including MDC1A.

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